Modeling Calcium-Induced Solubility in Caprine Milk Caseins Using a Thermodynamic Linkage Approach[1]

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ABSTRACT

The phenomena of calcium-induced precipitation of bovine and caprine whole caseins (salting out) and the resolubilization of these proteins at higher calcium concentrations (salting in) are thermodynamically linked with changes in protein solubility resulting from calcium binding. The differences in calcium sensitivities of caprine whole caseins under various conditions of temperature and ionic strength (KCl) appear to be correlated with the content of the α_{s1}-casein component. However, the solubility behavior of caprine whole caseins characterized by low content of α_{s1}-casein (5% of total) is more closely related to solubility properties displayed by bovine casein (38% of total). The properties of whole caprine casein high in α_{s1}-casein content (17% of total) appear to be dominated by the binding of calcium to higher affinity sites (phosphate groups), which results in less stability. Decreasing the temperature to 1°C dramatically altered the salting out of both caprine caseins but not bovine casein. These results suggested that the solubility and calcium-binding properties of caprine whole caseins are in part determined by hydrophobic interactions. However, salting out of both of the caprine caseins is effected by competitive K^+-Ca^{2+} binding at 1°C, indicating a role for ionic interactions as well. Because such KCl-dependent changes do not occur in whole bovine caseins, protein-protein interactions appear to be stronger in this case. These results show that alteration in casein composition can clearly effect the functionality of the whole casein and that thermodynamic linkage analysis can readily quantitate these differences that are linked to calcium binding.

(Key words: calcium binding, caprine casein, α_{s1}-casein, thermodynamic linkage)

Abbreviation key: **k** = salting-out or salting-in constants (used with 1 or 2, respectively).

INTRODUCTION

Problems in controlling physical properties of milks and dairy-based products can often be solved empirically, but long-term solutions require knowledge of the molecular structure. For instance, the solubility and colloidal stability phenomena of caseins have been studied for individual components and their calcium-binding capacity (8, 9, 15), but the casein micelle consists of a mixture of α_{s1}-, α_{s2}-, β-, κ-, and other minor casein components (10). Based upon studies of purified bovine milk proteins, α_{s1}-casein is theorized to be one of the most important structural components in the casein micelle. However, Raman spectroscopy experiments have shown that secondary structural differences exist between those observed for whole casein and those calculated from the sum of its purified component parts

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(5). Thus, the binding of calcium to the caseins (whole casein or its principal components, α_{s1}- and β-caseins), which is of fundamental importance in the formation of the micelle not only in vivo but also in food processing, could vary with altered casein composition as the result of protein-protein interactions. Indeed, for individual components, calcium binding is thermodynamically linked to both solubility and colloid stability (8, 9, 15). There is, then, much need to investigate such changes on complex protein systems, such as whole casein, where interactions among several casein components occur.

Caprine milk caseins have been the subject of controversy in that the level of α_{s1}-casein has been reported to be low or nonexistent (3, 6). However, considerable variation has now been reported (1, 11, 16, 17) for the content of α_{s1}-casein in caprine milk. This variation is important; Schmidt and Koops (24) have demonstrated that, for synthetic bovine micelles, alteration of the ratios of the various casein components dramatically changes the functionality of the system. Thus, an understanding of milk protein-protein interactions in the presence of calcium is important in the study of casein functionality and micelle stability. In order to begin to understand protein-protein and protein-salt interactions, these interactions need to be compared in systems containing salts. An equation based on the concepts of Wyman's theory of thermodynamic linkage (28) was recently proposed to quantify the linkage between calcium binding and resultant changes in solubility for individual bovine caseins (8, 9). This model has now been extended to quantify, for whole caseins, the degree of solubility related to calcium binding, to elucidate the interactions between calcium and caprine whole caseins characterized by low and high contents of the α_{s1}-casein component, and to compare these results with those found for whole bovine casein.

MATERIALS AND METHODS

Deionized Water

Deionized water, prepared by passage of distilled water over a mixed bed cation-anion exchanger, was used throughout this study.

Sources of Caseins

Whole caseins were prepared by isoelectric precipitation of the individual skim milk samples obtained from French-Alpine goats. The precipitate was dissolved by addition of NaOH to yield a solution of pH 7.0. The casein was reprecipitated, washed, and then resuspended. The sodium caseinate was subsequently cooled to 4°C and centrifuged at 100,000 × *g* for 30 min to remove residual fat. Finally, the suspension was dialyzed versus cold deionized water at 4°C for 72 h with three changes and then lyophilized.

The integrity of the samples was confirmed by SDS-PAGE, and densitometry was used to assess the relative concentrations of casein components (2).

Solubility of Caseins

Solubility of caprine caseins at 1 and 24°C was determined as follows. 1) Caseins (about 20 mg/ml) were dissolved in water and pH adjusted to 7.0 with .1*N* KOH or NaOH; the solution was then equilibrated in a water bath at the desired temperature for 20 min. 2) To 2 ml of protein solution (in thick-walled centrifuged tubes), 2 ml of $CaCl_2$ solutions, with or without buffer, and KCl were added. The tube was inverted and left to stand at the desired temperature for 30 min. 3) Tubes were centrifuged for 15 min at 43,654 × *g* at the desired temperature in an ultracentrifuge (model L-7; Beckman Instruments, Palo Alto, CA) with an SW 60 Ti swinging bucket rotor. 4) One milliliter of supernatant was transferred to a 10-ml volumetric flask containing 1 ml of N-sodium citrate plus a few milliliters of water and made up to volume with water. When solubility is determined at 1°C, pipettes must be prechilled to avoid precipitation of protein in the pipette. Concentrations were determined in 1-cm cuvettes at 280 nm; an absorptivity of .850 ml/mg cm at 280 nm was used for whole casein (22).

Theory and Data Analysis

Wyman's concept of linked functions (28) was useful for the treatment of the sequential precipitation (salting out) and resolubilization (salting in) of individual bovine casein components (8, 9). The best fit was found for a model

assuming two classes of binding sites for the ions responsible for the processes mentioned. The equilibria involved were formulated as

$$\underset{(S_0)}{p} + nx \overset{k_1^n}{\underset{}{\rightleftarrows}} \underset{(S_1)}{PX_n} + mx \overset{k_2^m}{\rightleftarrows} \underset{(S_2)}{PX_nX_m}, \quad [1]$$

where p is the unbound protein; x is the free salt; n and m are the number of moles of X bound to species PX_n and PX_nX_m; and S_0, S_1, and S_2 are the solubilities of the liganded species indicated. For this study, S_1 and S_2 will be relative to S_0. The mathematical relationship representing this stoichiometry can be represented according to

$$S_{app} = S_0f(p) + S_1f(PX_n) + S_2f(PX_nX_m), \quad [2]$$

where S_{app} is the apparent protein solubility at a given salt concentration (X_T), f(i) are the protein fractional components of species i, and the S are species previously defined. Incorporation of the salt-binding equilibrium constants (k_1 and k_2) as defined by [1] into [2] yields

$$S_{app} = \frac{S_0p}{p + k_1^npx^n} + \frac{S_1k_1^npx^n}{p + k_1^npx^n} + \frac{(S_2 - S_1)k_2^mpx^m}{p + k_2^mpx^m}, \quad [3]$$

where p is the concentration in percentage of the unbound protein, and x is the concentration of unbound salt. Cancellation of common terms yields

$$S_{app} = \frac{S_0}{1 + k_1^nx^n} + \frac{S_1k_1^nx^n}{1 + k_1^nx^n} + \frac{(S_2 - S_1)k_2^mx^m}{1 + k_2^mx^m}. \quad [4]$$

Equation [4] represents sequential binding. That is, $k_1 > k_2$ and the n sites become saturated with X prior to significant binding of X to the m sites. Also, for n or m > 1, k_1 and k_2 represent a mean value for each class of the n or m binding sites. In reality, n or m moles of salt will bind with only one equilibrium constant (K_1) (i.e., $K_1 = k_1^n$ and $K_2 = k_2^m$. The values of Equation [4] have so far been derived in terms of free salt; however, the equations can be extended to total salt concentration for two reasons: first, addition of Ca^{2+} causes the rapid polymerization of whole casein (27, 29) such that the number of particles present is small (a high degree of aggregation); second, corrections for binding, which can be done for purified caseins, altered our ability to use Equation [4] because the overall shapes of the plots remain constant, but their inflection points are shifted to lower values since the concentration of free calcium is lower than that of total calcium (15). Estimation of binding constants and the equation for the conversion to free salt have been given (15). This work compares three different caseins to suggest a practical end use, and thus total calcium is used throughout the study.

The model in Equation [4] was applied in the present study to the calcium-induced solubility profiles of caprine caseins. These solubility profiles were analyzed using an iterative nonlinear regression program (NLLSQ in BASIC) on a microcomputer that employed the Marquardt algorithm. This program minimizes the standard deviation of the experimental points from the curve, also known as the root mean square. The n and m values yielded the minimum root mean square value for the analysis with the minimum error in k_1 and k_2.

RESULTS

Calcium-Induced Solubility Profiles of Bovine and Caprine Caseins

Solubility profiles of whole bovine and caprine caseins in the presence of Ca^{2+} were studied; the soluble fraction was estimated after incubation for 20 min and centrifugation for 15 min at 43,700 × *g*. This centrifugation regimen was originally selected to test the solubility of purified caseins by clearing calcium caseinate precipitates from solution; in these cases, equilibrium was demonstrated between complexes and soluble phase (27). Here the method is adapted to whole casein, and each point represents a separate equilibrium. In these experiments, as the Ca^{2+} content is

raised in discrete increments, synthetic micelle formation occurs, and then a percentage of the micellar and nonmicellar caseins precipitates (salts out) under these conditions. At even higher Ca^{2+} concentrations, salting in occurs. The test, then, in essence measures changes in serum casein as a function of calcium concentration. Through variations of salt concentrations, or temperature, or both, the roles of ionic and hydrophobic interactions in the precipitating complexes can be dissected by quantitating their effects on salting out or salting in. Analyses of these results will be informative regarding the types of bonding that initially induce micelle formation and lead to precipitation and subsequent resolubilization.

Wyman's theory of thermodynamic linkage is based upon the concept that changes in an observable physical quantity (in this case solubility) can be linked to ligand binding. In previous studies on isolated caseins (8, 9, 15), the precipitation of the caseins in the presence of calcium was indeed shown to be linked to calcium binding, and calcium binding is the driving force in the precipitation reaction. This fact has been recognized by other workers (21, 27). Also important is that ligand binding, which is not linked to solubility, will not be disclosed by the method of analysis (e.g., β-casein binds calcium at 1°C, but the complex remains soluble). Equally important is that, for whole caseins, cooperative interactions may predominate, so that the integer values used to fit Equation [4] reflect mean numbers found in the regions in which changes in solubilities occur, and these are often not identical to the total number of calcium-binding sites in, e.g., equilibrium dialysis.

TABLE 1. Comparison of the percentage of casein distribution of bovine and caprine caseins by densitometry.

Casein type	Bovine	Low caprine α_{s1}-casein[1]	High caprine α_{s1}-casein[2]
α_{s2}-Casein	10.0	20.0	6.2
α_{s1}-Casein	38.0	5.0	17.1
β-Casein	40.0	48.0	50.1
κ-Casein	12.0	16.0	17.5

[1]This casein contained one of the lowest contents of α_{s1}-casein component in caprine milk found by Mora-Gutierrez et al. (18).

[2]This casein contained one of the highest contents of α_{s1}-casein component in caprine milk found by Mora-Gutierrez et al. (18).

Figures 1 and 2 show the Ca^{2+}-induced solubility profiles at 24°C of bovine and two caprine caseins. The two caprine caseins were selected as being high and low in α_{s1}-casein, recognizing that bovine casein is far above these two in α_{s1}-casein content (Table 1). All caseins show an initial precipitation (salting out), followed by a leveling off, which is interpreted as a salting in, the reversal of the precipitation process. In order to quantify the data, iterative nonlinear regression analyses (9, 15) were performed applying the concept of thermodynamic linkage. The data of Figures 1 and 2 were fitted with Equation [4], and the results are listed in Table 2.

The Ca^{2+}-induced solubility studies at 24°C on bovine casein (Figure 1) and caprine casein

TABLE 2. Calcium-induced solubility of caprine and bovine caseins at 24°C.[1]

Casein	k_1		S_1[2]		k_2		S_2[2]	
	— (L/mol) —		— (g/L) —		— (L/mol) —		— (g/L) —	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
Caprine								
Low in α_{s1}-casein[3]	68	12	2.5	1.5	2.8	.6	3.2	2.2
High in α_{s1}-casein[3]	137	13	.8	.2	4.4	.6	1.2	.3
Bovine[4]	24	1	1.1	.1	6.9	.6	3.3	.2

[1]Solutions buffered at pH 7.0, 10 m*M* imidazole·HCl, .07 *M* KCl.

[2]k_1 = Salting-out constant, k_2 = salting-in constant, S_i = the soluble protein species defined in Equation [1].

[3]n = 1, m = 2, where n and m are binding sites.

[4]n = 3, m = 2.

with low content of α_{s1}-casein (Figure 2A) appear to the eye to have solubility behaviors that are similar. However, the calculated values of k_1, the salting-out constant, were significantly different; the values of n, the apparent number of moles of Ca^{2+} bound to S_0 species to induce precipitation, were also different (Table 2). The larger k_1 obtained for the caprine casein with low content of the α_{s1}-casein component ($k_1 = 68 \pm 12$) compared with that for bovine casein ($k_1 = 24 \pm 1$), coupled with n = 1 for caprine casein, are all highly suggestive of linkage of solubility to binding sites having a more cooperative (concerted) character and a slightly higher affinity for Ca^{2+} in caprine caseins. The high caprine α_{s1}-casein had the greatest k_1 (137 ± 13); with n = 1, this curve is indicative of more cooperative interactions in caprine caseins. Because thermodynamic linkage discloses the nature of only those binding sites that lead to a change in the physical parameter measured (in this case solubility), the following interpretation can be offered for the differences observed in Table 2. All caseins first bind calcium and form synthetic micelles. The caprine casein high in α_{s1}-casein content appeared to be salted out at a relatively lower calcium concentration (7 m*M* is the concentration for one-half precipitation, calculated from $1/k_1$). Thus, the high caprine α_{s1}-casein caseinate may not form synthetic micelles that are stable over the broad range of calcium ion concentrations occurring in milk (10). However, the low caprine α_{s1}-casein and the bovine casein are half salted out at 15 and 42 m*M*, respectively, which is above the calcium ion concentrations in milk; these caseins are predicted to exhibit increased stability for their synthetic micelles.

The salting-in constants presented in Table 2 differ markedly in magnitude from the respective salting-out constants. Salt binding to higher affinity sites induces precipitation, and then lower affinity Ca^{2+} sites (as inferred from the small k_2 values) would be involved in binding, which leads to resolubilization of these milk proteins at higher salt concentrations (140 to 300 m*M*). The value of the salting-in constant for bovine casein ($k_2 = 6.9 \pm .6$ L/mol) is larger than those of either caprine casein. One would expect that the presence of secondary binding sites with higher affinity for Ca^{2+} would lead to a more solubilized bovine casein at higher Ca^{2+} concentrations. However, caprine casein with low content of α_{s1}-casein is predicted by Equation [4] to be salted in to the same extent as bovine casein (Table 2, S_2). In the case of bovine β-casein, even though Ca^{2+} binds to the protein (21), the complexes are cold-soluble because of

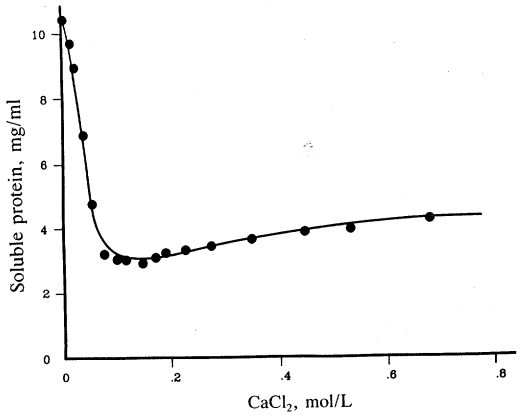

Figure 1. Solubility of 24°C of bovine casein as a function of increased $CaCl_2$ concentrations. Solutions buffered at pH 7.0, 10 m*M* imidazole·HCl with .07 *M* KCl. The experimental data, done in triplicate, were averaged and fitted with Equation [4] by nonlinear regression analysis. Results of analyses are in Table 2.

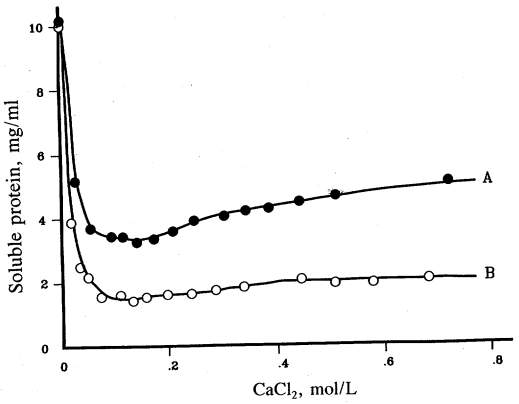

Figure 2. Solubility at 24°C of caprine casein with A) low and B) high content of the α_{s1}-casein as a function of increasing $CaCl_2$ concentrations. Solutions buffered at pH 7.0, 10 m*M* imidazole·HCl with .07 *M* KCl. The experimental data, done in triplicate, were averaged and fitted with Equation [4] by nonlinear regression analysis. Results of analyses are given in Table 2.

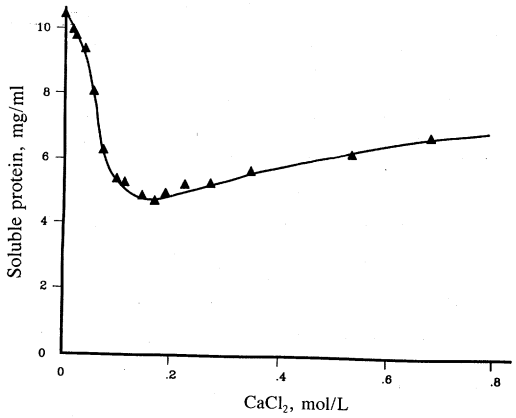

Figure 3. Solubility at 1°C of bovine casein as a function of increasing $CaCl_2$ concentrations. Solutions were buffered at pH 7.0, 10 m*M* imidazole·HCl with .07 *M* KCl. The experimental data, done in triplicate, were averaged and fitted with Equation [4] by nonlinear regression analysis. Results of analyses are in Table 3.

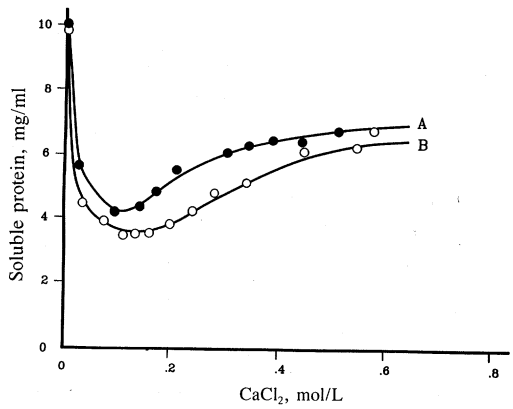

Figure 4. Solubility at 1°C of caprine casein with A) low and B) high content of the α_{s1}-component as a function of increasing $CaCl_2$ concentrations. Solutions buffered at pH 7.0, 10 m*M* imidazole·HCl with .07 *M* KCl. The experimental data, done in triplicate, were averaged and fitted with Equation [4] by nonlinear regression analysis. Results of analyses are given in Table 3.

their highly hydrophobic nature; it was therefore of interest to study the solubility profiles of the caprine caseins at 1°C because β-casein is the predominant casein (23) in these milks (Table 1).

Effect of Reduced Temperature on Calcium-Induced Solubility

Calcium-induced solubility was also studied at 1°C; data of Figures 3 and 4 were analyzed according to Equation [4] using nonlinear regression analysis. The final values with the corresponding standard errors of the salting-out constant, k_1; the salting-in constant, k_2; the minimum solubility (S_1); and the predicted solubility (S_2), which occurs after salting in, are presented in Table 3.

Changes occurring in the solubility and related properties of proteins as a result of increasing concentrations of salts have been mainly attributed to effects upon electrostatic interactions and hydrophobic bonding (9, 26). By studying these solubilities at 1°C, at which temperature hydrophobic effects are minimized, solubility due to binding and thus salting out, as well as electrostatic salting in effects, can be assessed. Results from the pres-

TABLE 3. Calcium-induced solubility of caprine and bovine caseins at 1°C.[1]

Casein	k_1		S_1[2]		k_2		S_2[2]	
	— (L/mol) —		— (g/L) —		— (L/mol) —		— (g/L) —	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
Caprine								
Low in α_{s1}-casein[3]	63	10	2.9	.4	5.1	.4	3.7	.3
High in α_{s1}-casein[3]	114	9	2.9	.1	2.5	.1	5.2	.4
Bovine[4]	15	1	1.0	.1	5.3	.8	6.4	.6

[1]Solutions buffered at pH 7.0, 10 m*M* imidazole·HCl, .07 *M* KCl.

[2]k_1 = Salting-out constant, k_2 = salting-in constant, S_i = the soluble protein species defined in Equation [1].

[3]n = 1, m = 3, where n and m are binding sites.

[4]n = 3, m = 1.

ent study suggested that increasing concentrations of $CaCl_2$ at 1°C (Figures 3 and 4, respectively) had two distinct effects upon the solubility properties of the caseins. At low calcium concentrations (approximately .05 to .10 *M*), all caseins were salted out (presumably by calcium binding), as evidenced by significant decreases in protein solubility (S_1; Table 3). Addition of $CaCl_2$ at concentrations greater than that necessary for synthetic micelle formation, tends to neutralize electrostatic repulsion and thus favor a reduction in solubility even though hydrophobic associations are minimized at 1°C. At higher $CaCl_2$ concentrations (>.10 *M*), resolubilization of the caseins occurs. This phenomenon has been ascribed for purified α_{s1}-casein to increased charge of the calcium-protein complex (9, 27) (i.e., reversal to a net positive charge as a consequence of cation binding).

The generally larger k_1 values obtained in the presence of calcium for caprine casein high in the α_{s1}-casein compared with those of caprine casein low in the α_{s1}-casein and bovine casein (Tables 2 and 3) indicate a higher salt sensitivity of this type of caseinate regardless of temperature. Thus, these micelles are least stable to added calcium ions, even in cold temperatures. Mora-Gutierrez et al. (19) found that purified caprine α_{s1}-casein is more cold-soluble than bovine α_{s1}-casein B. This result, coupled with the predominance of the cold-soluble β-casein in caprine caseins and studies of isolated casein components (8, 9, 15), predicts much higher calcium solubilities for caprine casein at 1°C than were observed in the present study. That protein-protein interactions play a role is evident; Byler and Farrell (5) observed increased structural components for whole caseins over those summed from their components by infrared spectroscopy. The differences in salt sensitivities of the caprine caseins represented in Figures 1 through 4 may thus be explained by compositional differences, in particular the α_{s1}-casein component, which may alter solubility properties through concentration dependent protein-protein interactions with κ-casein.

The two other parameters obtained by this analysis are n and m. The n and m values are the same, n = 1 and m = 3 at 1°C and n = 1 and m = 2 at 24°C for both caprine caseins. The relatively low values for n and m should not be interpreted literally as only a simple binding site, because it is well known that multiple-binding sites with exactly the same equilibrium constant yield only a single binding isotherm (9, 15). Hence, a value of n or m represents a class of protein-binding sites perhaps even acting in a cooperative fashion rather than a single binding site linked to the solubility change of the protein; that is, the larger n or m is, the greater the number of sites acting independently. Moreover, the amount of β-casein is higher in caprine caseins, which suggests that the whole caprine caseins have lower net negative charge than the bovine casein at the pH conditions of milk, and therefore could bind, overall, less calcium under our experimental conditions. At 1°C, the degree of polymerization is much lower, and more potential sites for calcium binding may be exposed. The observed increase in m values at 1°C (Tables 2 and 3) clearly indicates that both caprine caseins expose a higher number of weak secondary sites for calcium binding as a result of decreased casein self-association. However, k_1 is still much greater for the high caprine α_{s1}-casein, even at 1°C. If indeed k_1 and k_2 are constants linked to solubility, then effects of salt on these constants can be tested. For example, K^+ could compete with Ca^{2+} and thus provide knowledge regarding relative bond strengths in the precipitates, particularly at 1°C.

Effect of Ionic Strength on Calcium-Induced Solubility

Figure 5 represents the effect of varying the ionic strength (KCl) on the calcium-induced solubility profiles for caprine caseins with low or high content of α_{s1}-casein, respectively. The analyses of the data are summarized in Tables 4 and 5. The calcium-induced precipitation of the caprine caseins diminishes with an increase in ionic strength; S_1 increases with increased KCl. This increase indicates that electrostatic interactions are important in binding of the calcium, which is thermodynamically linked to the solubility of the caseins under the conditions studied. Electrolytes tend to weaken salt linkages by producing a stabilizing Debye-Hückel atmosphere around the charged groups when they are in the dissociated form (14), and a general electrostatic effect resulting from

competition between Ca^{2+} and K^+ for negative protein-binding sites was suggested (8, 9) as the mechanism responsible for the increased solubility of bovine α_{s1}-casein A at high ionic strengths (KCl).

The general decrease of k_1 values upon increasing concentration KCl is highly suggestive of displacement of Ca^{2+} by K^+ from binding sites on these proteins. Interestingly, no changes in k_1 occurred with changes in KCl for whole bovine casein (19). Here, then, is the major difference between the bovine and caprine caseins. The bovine caseins bind calcium, form synthetic micelles, and precipitate; their calcium-protein and protein-protein interactions are far more stable than those of both caprine caseins at 24 and at 1°C over the concentrations of KCl used in these studies. The caprine caseins are less stable in general, and the k_1, or salting-out constants, are more sensitive to KCl at 1°C. Such behavior is in line with a higher degree of hydrophobic interactions in the caprine caseins. Plots of k_1 for the two caprine and bovine caseins as a function of KCl are given in Figure 6, which shows that k_1 values for both caprine caseins are high in the absence of KCl and then decrease with added salt.

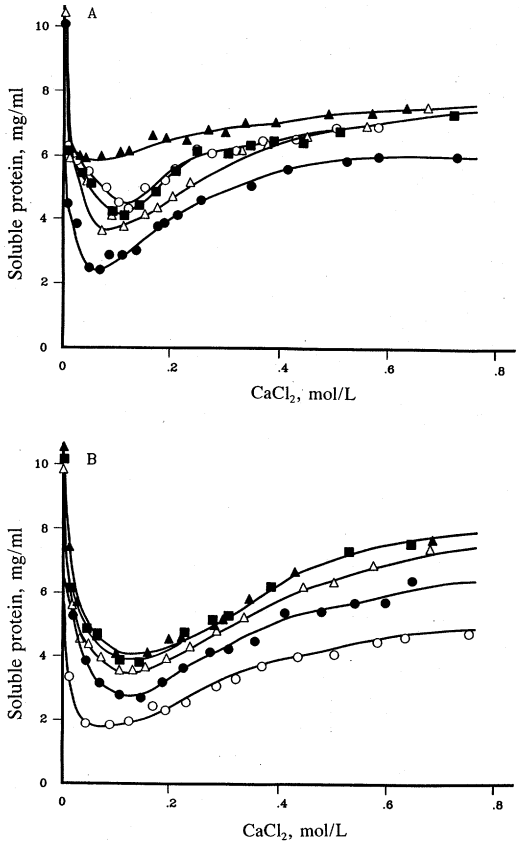

Figure 5. Solubility at 1°C of caprine casein with A) low and B) high content of the α_{s1}-component as a function of increasing $CaCl_2$ and KCl concentrations. Key: ▲, .140 *M* KCl; ○, .105 *M* KCl; ■, .07 *M* KCl; △, .035 *M* KCl; ●, no buffer or KCl. (B) Key: ■, .140 *M* KCl; ▲, .105 *M* KCl; △, .07 *M* KCl; ●, .035 *M* KCl; ○, no buffer or KCl. The experimental data, done in triplicate, were averaged and fitted with Equation [4] by nonlinear regression analysis. Results of analyses are given in Tables 4 and 5.

The plots of Figure 6 were analyzed with Equation [4]. The k_1 values for the high caprine α_{s1}-casein decrease to a limiting value with a calculated constant for the transition of 240 L/mol. The k_1 values for the low caprine α_{s1}-casein also decrease with a constant for the transition equal to 27. Also different for the low caprine α_{s1}-casein protein is its behavior at increased KCl concentrations. Here, the low caprine α_{s1}-casein has a k_1 that approaches that of bovine casein (mean = 15 ± 2 L/mol) and then rises dramatically. The calculated constant for this second transition in 8 L/mol. It is noteworthy, from the large k_1 values for the caprine caseins in the absence of KCl (Tables 4 and 5), that a decrease in electrostatic repulsion leads to considerable protein precipitation because of the binding of calcium to the protein. Addition of KCl decreases k_1, so the simplest interpretation appears to involve competitive K^+/Ca^{2+} binding. However, for the low caprine α_{s1}-casein this trend is reversed at elevated KCl; whether this trend is caused by K^+ or Cl^- is uncertain. However, β-casein is the predominant protein in the caprine casein. Thompson et al. (25) observed large increases in the degree of calcium-induced hydration of bovine β-casein at elevated KCl concentrations. Here, for the low α_{s1}-casein (highest β-casein), increased salt binding may lead to dramatically changed solvation and concomitant increase in k_1. For the high caprine α_{s1}-casein, this change does not occur perhaps because of its increased α_{s1}-casein. The estimated S_2 values reflect the considerable electrostatic repulsion as a consequence of the binding of K^+-Ca^{2+} to the low caprine α_{s1}-casein. The S_2 values for high α_{s1}-casein are relatively unaffected by KCl as are the S_2 of bovine casein (19). The overall differ-

TABLE 4. Ionic strength dependence of calcium-induced solubility of caprine casein low in α_{s1}-casein at 1°C.

KCl	k_1		S_1[1]		k_2		S_2[1]	
	— (L/mol) —		— (mg/ml) —		— (L/mol) —		— (mg/ml) —	
(m*M*)	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
0[2]	210	37	1.0	.1	4.5	.3	5.0	.3
35[3]	113	7	2.6	.1	3.8	.09	5.3	.2
70[4]	61	11	2.7	.5	5.1	.4	3.9	.6
105[5]	30	3	4.0	.1	3.8	.2	3.9	.5
140[6]	133	8	5.9	.1	3.8	.4	1.8	.1

[1] k_1 = Salting-out constant, k_2 = salting-in constant, S_i = the soluble protein species defined in Equation [1].

[2] n = 1, m = 2, where n and m are binding sites.

[3] n = 1, m = 3.

[4] n = 1, m = 3.

[5] n = 1, m = 3.

[6] n = 8, m = 2.

ence for the calcium-induced solubility behavior of both caprine caseins at several ionic strengths (KCl) (Figure 5) seems to reside in the relative contents of casein. The presence of high concentrations of β-casein should make the caprine caseins extremely soluble and unresponsive to calcium precipitation at 1°C, but some stronger protein-protein interactions overcome this anticipated effect, particularly for the high α_{s1}-casein caseinates. The sensitivity of the k_1 values of caprine caseins to KCl reflect overall weaker protein-protein interactions than for bovine casein. Moreover, little change in the value of k_2, the salting-in equilibrium constant, is observable for either caprine casein as a consequence of increasing ionic strength (KCl) in Tables 4 and 5. However, for the caprine casein low in α_{s1}-casein at all ionic strengths, the k_2 values decrease and are somewhat higher than the corresponding estimates for caprine casein high in α_{s1}-casein.

DISCUSSION

This study focuses on the effects of calcium binding on the solubility of whole caseins and compares three caseins with varying ratios of α_{s1}-, α_{s2}-, β-, and κ-casein components. Cal-

TABLE 5. Ionic strength dependence of calcium-induced solubility of caprine casein high in α_{s1}-casein at 1°C.

KCl	k_1		S_1[1]		k_2		S_2[1]	
	— (L/mol) —		— (mg/ml) —		— (L/mol) —		— (mg/ml) —	
(m*M*)	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
0[2]	276	28	1.1	.2	3.2	.3	4.1	.5
35[3]	87	9	1.8	.2	3.5	.1	4.0	.4
70[4]	114	9	2.9	.1	2.5	.1	5.2	.4
105[5]	78	7	3.5	.2	2.7	.1	4.4	.4
140[6]	77	15	3.1	.4	2.8	.3	5.1	1.0

[1] k_1 = Salting-out constant, k_2 = salting-in constant, S_i = the soluble protein species defined in Equation [1].

[2] n = 1, m = 2, where n and m are binding sites.

[3] n = 1, m = 3.

[4] n = 1, m = 3.

[5] n = 1, m = 4.

[6] n = 1, m = 3.

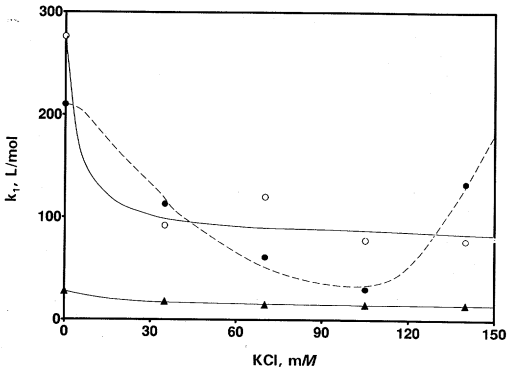

Figure 6. Replots of salting-out constant (k_1) values as a function of KCl concentration (millimolar) obtained for the calcium-induced precipitation of the low (●) and high (○) caprine α_{s1}-caseins at 1°C (Tables 4 and 5). Bovine casein (▲) is shown for comparison (19).

cium binding to individual species has been studied in detail, and the apparent mean association constants range from 400 to 1400 L/mol. These values are compared as log K_A in Table 6. Several lines of evidence point to the fact that, for individual caseins, these are indeed mean constants because they can be dissected under experimental conditions (21). In general, this result has been attributed to nonequivalent phosphate sites or to cooperativity, whereby the binding of the first calcium enhances that of the second. From the perspective of the end use of whole caseins in the presence of calcium, it is uncertain whether or not these studies on isolated proteins directly apply because individual caseins are historically separated by urea and thus the neighboring casein components in unfractionated whole casein may or may not be these found in aggregated isolated caseins (13).

In experiments with whole casein, addition of calcium ions results in binding, and older studies (29) show the mean log K_A values to be in some agreement with those of purified caseins (Table 6). For whole casein, a progression of events can be envisioned as a result of calcium binding. Jang and Swaisgood (13) demonstrated very high affinity binding to submicelles with log K_A = 4.2. This binding could represent the very first set of sites bound prior to colloid formation. Next, binding to lower affinity sites produces colloid formation. All three samples of whole casein used in this study were roughly similar in colloid stability

TABLE 6. Comparison of salting-out and salting-in constants with association constants for calcium binding to and precipitation of caseins.

Casein or model	log k_1[1]	log k_2	log K_A	log $1/[Ca^{2+}]_{crit}$
α_{s1}-Casein A, bovine	2.20[2]	1.02[2]	. . .	. . .
α-Casein B, bovine	2.26[2]	.39[2]	2.6[3], 2.96[4]	2.55[4]
α_{s1}-Casein, caprine	2.02[5]	.95[5]	. . .	. . .
β-Casein, bovine	2.19[2]	. . .	2.92[4]	2.25[4]
Whole casein, bovine	1.38[5]	.84[5]	4.26[6], 3.37[7] 2.77[7]	
Whole casein, caprine–high	2.14[8]	.64[8]	. . .	. . .
Whole casein, caprine–low	1.83[8]	.44[8]	. . .	. . .
o-Phosphoserine	. . .	. . .	2.23[3]	. . .
Glutamate	. . .	. . .	.83[3]	. . .

[1]k_1 = Salting-out constant, k_2 = salting-in constant, K_A = association constant, and $[Ca^{2+}]_{crit}$ = critical concentration of Ca^{2+} at which casein is precipitated; all constants are expressed in liters per mole.

[2]Farrell et al. (9).

[3]Dickson and Perkins (7).

[4]Parker and Dalgleish (21).

[5]Mora-Gutierrez et al. (19).

[6]Jang and Swaisgood (13).

[7]Zittle et al. (29).

[8]This study.

(20). After saturation of colloid-inducing sites, further binding resulted in precipitation (destabilization); here again the three caseins studied were similar in their destabilization as viewed by the colloid stability test (20). In this work, we have applied the functional test of solubility to attempt to discriminate between the three samples. Because of the higher centrifugal fields, colloidal and calcium-caseinate complexes are salted out, and, as the calcium or KCl contents are increased, salting in occurs. Earlier work on α_{s1}-casein A demonstrated that salting out and salting in for calcium caseinate were driven by calcium binding (9). We have thus attempted to extend the theory of thermodynamic linkage as developed by Wyman to this functional test (solubility) for whole caseins to compare their properties.

In general, the concept of thermodynamic linkage is to analyze an observable physical phenomenon that is driven by binding through the use of binding isotherms. The resultant binding constants, in our case k_1 and k_2, reflect the concentration of ligand at the half point for changes in solubility. However, these constants are linked to the selected binding, which results in the physical change. Clearly, binding to β-casein at 1°C (21) does occur, but, because the protein is soluble, no thermodynamic linkage to solubility occurs. Because purified caseins, as noted, have unequal binding sites, the salting-out constant (i.e., the final binding leading to insolubility through charge neutralization) is expected to not be equal to the mean binding constant, nor should it be influenced by environmental conditions (KCl or temperature) in the same fashion as the mean binding constant either for a purified component or whole casein.

Comparison of the k_1 and k_2 solubility constants with binding constants is made in Table 6. In general, the log k_1 values for salting out are always lower than the average binding constants when k_1 values are derived from total calcium contents. When binding is taken into account (15), the k_1 values are higher. For example, log k_1 for α_{s1}-casein B increases from 2.26 to 2.47, which is closer but still not equal to log K_A (21). Parker and Dalgleish (21), during their studies of calcium binding, reported a calculated constant termed Ca^{2+}-crit, the critical concentration of calcium at which precipitation begins. The log $1/[Ca^{2+}]_{crit}$ values for this factor are also given in Table 6. Substantially better agreement between these values for bovine α_{s1}- and β-caseins and k_1 are seen in Table 6. The Ca^{2+}-crit factor was calculated during direct calcium-binding studies (21); here we have computed k_1 from solubility data. These two constants computed in very different ways most likely are related to the same phenomenon, the calcium-induced precipitation of casein. The simpler solubility functionality test computed through thermodynamic linkage can thus provide a useful salting-out constant. Hence, the calcium ion concentration at $1/k_1$ is 42, 15, and 7 m*M* for bovine, caprine low, and caprine high, respectively, and these values provide an insight into the overall concentrations at which these systems are soluble, as well as predicting the extent of maximum solubility (S_1 and S_2).

The k_2 is related to salting in. Studies of this term were conducted at 1°C, at which temperature hydrophobic interactions are minimized. For purified α_{s1}-caseins A and B, these values represent additional calcium binding leading to resolubilization, and, in the case of α_{s1}-casein A, this was measured directly in electrophoretic experiments where the protein became cationic (9). Calcium binding is again hypothesized as the driving force for salting in. Thus, k_2 values derived from thermodynamic linkage represent the concentration of calcium at half salting in and are a reflection of the binding leading to increased solubility. In general, the k_2 values reflect binding to weaker sites, probably carboxyl, as seen from Table 6; such calcium-carboxyl interactions have been inferred from Fourier transform infrared measurements (5).

CONCLUSIONS

In summary, the differences in calcium solubilities of caprine whole caseins under various conditions of temperature and ionic strength (KCl) appear to be correlated with the content of the α_{s1}-casein component. However, the solubility behavior of caprine whole caseins characterized by low content of α_{s1}-casein (5% of total) is more closely related to solubility properties displayed by bovine casein (38% of total). Decreasing the temperature to 1°C dramatically altered the salting out of both caprine caseins but not bovine casein.

These results suggested that the solubility-related, calcium-binding properties of caprine whole caseins are in part determined by the hydrophobic interactions of the dominant protein, β-casein, which has a strong salt dependency for both binding and hydration (21, 25, 27). However, salting out of both of the caprine caseins is effected by competitive K^+-Ca^{2+} binding at 1°C, indicating a role for ionic interactions as well. Because KCl-dependent changes do not occur in bovine caseins, protein-protein interactions appear to be stronger in bovine casein where α_{s1}-casein predominates; this result could be due to stronger concentration-dependent α_{s1}-casein-κ-casein interactions. These results clearly show that alteration in casein composition can effect the functionality of the whole casein and that thermodynamic linkage analysis can readily quantitate these differences, which are linked to calcium binding.

The new findings provide a framework for further testing and exploration of the effect of caprine milk casein composition in structural and energetic terms. Current studies of the effects of salts on water-binding capacity of caprine caseins appear promising. As the casein system continues to be a prototype for attempts to understand other complex protein mixtures, studies of the individual subunits in those systems may also help to interpret the effects of salts on protein association and dissociation.

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